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Two Types of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone Hemoglobin Adducts, from Metabolites Which Migrate into or Are Formed in Red Blood Cells[1]

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ABSTRACT

The tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), is considered to play an important role in the induction of lung cancer in tobacco users. In rats treated with [5-^{3}H]NNK, 20 to 40% of the tritium bound to hemoglobin (Hb) is released by base hydrolysis as 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB). This HPB-releasing adduct has been quantified in tobacco users and is considered a biochemical marker for uptake and activation of tobacco-specific nitrosamines. In this paper we report the formation of this adduct in red blood cells (RBC) cultured for 2 h with hepatocytes and 5 μM NNK (6.35 ± 0.21 fmol HPB/mg Hb). The HPB-releasing adduct was not formed in RBC in the absence of hepatocytes (<0.5 fmol/mg Hb). Therefore, the HPB-releasing adduct must form from a pyridyloxobutylating metabolite of NNK *which traveled out of the hepatocytes and into RBC where it reacted with Hb.* Other distinct Hb adducts were formed when NNK was incubated with RBC alone. 4-Oxo-4-(3-pyridyl)butyric acid was detected by radio flow high-performance liquid chromatography in the media of these incubations. The Hb isolated from RBC incubated with [C^3H_3]NNK contained as much as 10 times more covalently bound tritium than the Hb from [5-^{3}H]NNK-treated cells. [C^3H_3]-1-Methylhistidine and [C^3H_3]-*S*-methylcysteine were formed when [C^3H_3]NNK was incubated with the 25,000 × *g supernatant from RBC. This supernatant contains 50 mg Hb/ml. We* propose that Hb mediates α hydroxylation of NNK at the methylene carbon. The α-hydroxynitrosamine formed decomposes to methanediazohydroxide and 4-oxo-1-(3-pyridyl)butanal. The former would methylate nucleophilic sites in Hb, *i.e.*, cysteine and histidine. The latter would bind to Hb or be further oxidized to 4-oxo-4-(3-pyridyl)butyric acid. The ability of the RBC to activate NNK to Hb-binding species stresses the importance of understanding how a particular adduct is formed prior to its use as a biochemical marker or internal dose monitor.

INTRODUCTION

Carcinogen hemoglobin adducts have been proposed as biochemical markers which may be used to measure the internal dose of an activated carcinogen (1–3). Thus, hemoglobin adducts act as surrogate measures for DNA adducts. This relationship, between DNA and hemoglobin adducts, assumes that the reactive metabolite which binds to each of these macromolecules is the same, or that the formation of one correlates with the other. The simplest mechanism is that the carcinogen is activated in one tissue, for example the liver, and the metabolite formed then enters the RBC.

Hemoglobin adducts of NNK[3] have been proposed as biochemical markers of the ability of an individual tobacco user to activate this lung carcinogen (4–6). NNK is a tobacco-specific nitrosamine which is activated by α hydroxylation in a number of different tissues and species (7–10). The hydroxynitrosamines *1* and *2* formed, decompose to methanediazohydroxide *6* and 4-oxo-4-(3-pyridyl)-butanediazohydroxide *3* and the corresponding aldehydes *4* and *5* (Fig. 1). The diazohydroxides react with both DNA and hemoglobin. In rats treated with [5-^{3}H]NNK, 20–40% of the tritium bound to hemoglobin is released upon hydrolysis with base as HPB (4, 11). The HPB-releasing adduct is an ester of aspartic or glutamic acid (12). This adduct has been quantified in tobacco users, and is considered a biochemical marker for uptake and activation of carcinogenic tobacco-specific nitrosamines (6).

In this paper, we describe the use of an *in vitro* system composed of a monolayer of hepatocytes and RBC suspended in the culture media to study the mechanism of formation of the HPB-releasing adduct in hemoglobin. The data presented here provide evidence that this adduct is formed by a metabolite of NNK produced in hepatocytes, and transported into RBC. In addition, we observed the formation of other NNK hemoglobin adducts in the absence of hepatocytes. That is, incubation of RBC alone with [5-^{3}H]NNK resulted in the generation of [5-^{3}H]NNK metabolites which were covalently bound to hemoglobin. The pathway by which this may occur was investigated.

MATERIALS AND METHODS

Chemicals. [5-^{3}H]NNK (2.1 or 1.84 Ci/mmol) and [C^3H_3]NNK (1.06 Ci/mmol) were purchased from Chemsyn Science Laboratories (Lenexa, KS). [5-^{3}H]NNK contains tritium in position 5 of the pyridine ring. NNK metabolites were synthesized as previously described (13, 14). Williams' medium was purchased from Flow Laboratories (Rockville, MD). β-Glucuronidase Type IX-A and all methyl amino acids were obtained from Sigma Chemical Co. (St. Louis, MO).

Isolation of RBC and Hemoglobin Solutions. Six to 10 ml of blood were drawn from a volunteer and collected in an EDTA-containing tube (Vacutainer, Becton Dickinson, Rutherford, NJ). RBC were pelleted at 900 × *g* and the supernatant and buffy coat were removed. The RBC pellet was washed 3 times in 0.9% NaCl solution. After the final wash, the pellet was resuspended in a volume of 0.9% NaCl solution equal to the original volume of blood. Care was taken to ensure that no WBC were present in the final RBC pellet. In several instances the RBC fraction was obtained by using a colloidal silica density gradient (Sepracell-MW, Sepratech, Oklahoma City, OK). The density gradient allows complete separation of the WBC and RBC. The use of RBC purified in this manner did not change the outcome of any of the experiments described below. Hemoglobin solutions were prepared from frozen RBC pellets as previously described (4). Briefly, the RBC are lysed in H_2O, an equal volume of 0.67 M potassium phosphate buffer (pH 6.5) is added, and the resulting solution is centrifuged at 25,000 × *g* for 25 min. The supernatant, containing hemoglobin, is dialyzed against several changes of H_2O. To study the ability of hemoglobin to activate NNK, a freshly prepared 25,000 × *g* supernatant from rat or human RBC was used. The concentration of hemoglobin was determined as cyanomethemoglobin (Sigma Kit 525A).

Isolation and Culturing of Hepatocytes. Hepatocytes were isolated from male F344 rats (Charles River, Kingston, NY) by collagenase perfusion as described previously (15). Viability, as measured by trypan blue exclusion, was from 85 to 90%. The hepatocytes were plated at a density of 3 × 10^6 cells/culture dish (100 mm) in Williams' media and cultured for 2–3 h under an atmosphere of 95% O_2:5% CO_2 at 37°C. After 2–3 h, essentially all the hepatocytes were bound to the plate. The medium was then replaced with fresh Williams' medium containing 5 μM [5-^{3}H]NNK.

Metabolism of NNK by Hepatocytes, Hepatocytes plus RBC, and RBC Alone. Two groups of culture dishes containing 3 × 10^6 hepatocytes prepared as above were incubated at 37°C under an atmosphere of 5% CO_2:95% O_2, with rocking (10 rpm). The dishes in one group contained 6 ml Williams'

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[3] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-butanol; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; OPB, 4-oxo-1-(3-pyridyl)-1-butanone; OPBA, 4-oxo-4-(3-pyridyl)butyric acid; NDMA, *N*-nitrosodimethylamine; HPLC, high-performance liquid chromatography.

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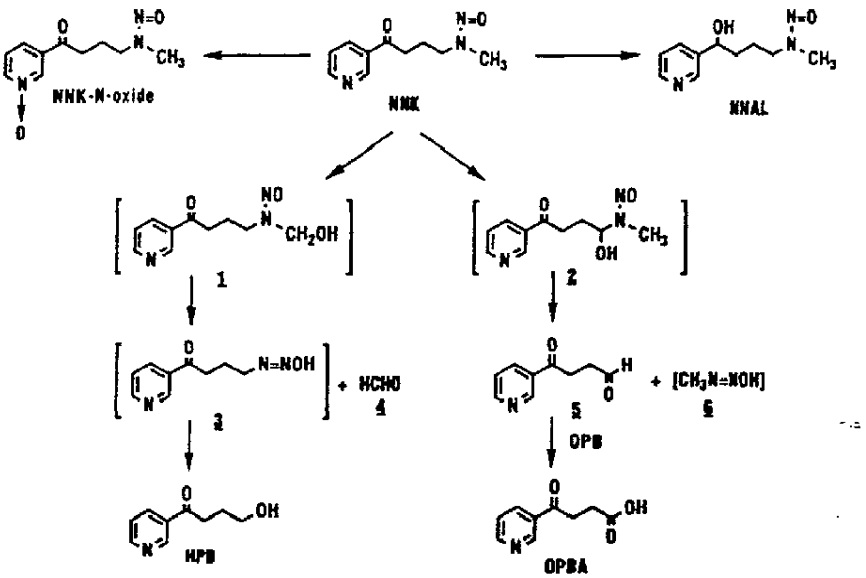

Fig. 1. Major NNK metabolism pathways.

medium with 5 μM [5-^{3}H]NNK. The second group contained 5 ml Williams' media and 1 ml of a freshly prepared RBC suspension with 5 μM [5-^{3}H]NNK. A third group of dishes contained 5 ml Williams' media, 1 ml RBC with 5 μM [5-^{3}H]NNK, but no hepatocytes. With each experiment, a control dish was incubated which contained only [5-^{3}H]NNK and media. After 2 to 18 h of incubation the medium, or the medium containing RBC, was removed from the plate. The RBC were pelleted by centrifugation at 900 × *g*. The supernatants and the media from dishes containing hepatocytes only were frozen at −20°C until analysis. The pellet was washed 4–5 times in 6 ml of 0.9% NaCl solution. The final wash contained less than 200 dpm/ml. The RBC pellet was stored at −20°C until analyzed.

Analysis of NNK Metabolites. The medium from each of the culture dishes was mixed with reference metabolites and analyzed by HPLC with radio flow detection (Flow-one/beta detector, Radiomatics Packard Instrument Co., Meridian, CT). The metabolites were separated on a Phenomonex Bondclone 10 C_{18} column (300 x 3.9 mm, Torrance, CA) with a linear gradient from 100% solvent A (20 mM sodium phosphate buffer, pH 7.0) to 70% A:30% methanol in 60 min, flow 1 ml/min (system I). A second HPLC system (II) used a Whatman Partisphere C_{18} column (Krackler Scientific, Inc., Albany, NY). Metabolites were eluted with a linear gradient from 100% solvent A to 92% solvent A:8% methanol in 16 min, followed by 15 min at 92% solvent A:8% methanol, then a second linear gradient to 30% methanol in 39 min. The flow was 1 ml/min. A third system (III) was used to confirm the identity of OPBA (16). This metabolite was collected from system I, then injected on a Bondclone 10 C_{18} (300 x 3.9 mm, Phenomonex) column and eluted with 10-min isocratic flow, 100% 20 mM sodium acetate, pH 4.5 (solvent A), followed by a linear gradient from 100% solvent A to 50% solvent A:50% solvent B (50% methanol:H_2O) in 50 min. HPB and OPB coelute in system I. To determine the relative amounts of each in this peak, it was collected and derivatized with sodium bisulfite. The HPB and OPB bisulfite adduct were then separated by HPLC analysis as described previously (17).

Activation of [5-^{3}H]NNK and [C^3H_3]NNK by Hemoglobin. Four ml of Williams' media and 2 ml of a freshly prepared hemoglobin solution (the 25,000 × g supernatant containing 49.6 mg hemoglobin/ml) and either 5 μM [C^3H_3]NNK or 5 μM [5-^{3}H]NNK were mixed in a 100-mm culture dish. The mixture was incubated with rocking (10 rpm) at 37°C under an atmosphere of 5% CO_2:95% O_2 for 18 h. The hemoglobin solution was then dialyzed extensively against 500 ml H_2O (4 times, 2 h each, and once for 18 h) to remove unbound NNK and NNK metabolites.

Analysis of Hemoglobin for Total Tritium and [^{3}H]HPB. Globin was isolated from [5-^{3}H]NNK- or [C^3H_3]NNK-treated hemoglobin, and hemoglobin obtained from [C^3H_3]NNK- or [5-^{3}H]NNK-treated RBC, by precipitation with acidic acetone (4, 18). RBC and hemoglobin solutions obtained from F344 rats as well as from humans were used as noted. Prior to precipitation of globin any unbound tritiated NNK or metabolites were removed by extensive dialysis. The absence of unbound [^{3}H]HPB in the hemoglobin solution was confirmed by applying a portion of the solution to a Centricon centrifugal microconcentrator (Amicon, Beverly, MA) and analyzing the filtrate by radio flow HPLC, using system I. The precipitated globin was washed with ice-cold acetone and dried in an oven overnight at 30°C.

To determine total tritium bound, 5–10 mg globin were dissolved in 1 ml 0.01 N HCl. The average radioactivity present in 3 aliquots was determined by liquid scintillation counting in Monofluor (National Diagnostics, Somerville, NJ). The protein content of the globin solution was determined by using the Bio-Rad (Richmond, CA) protein assay, and the extent of binding was expressed as fmol total metabolites bound per mg globin. The [^{3}H]HPB released from the globin was determined as described previously (11). Briefly, globin (~100 mg) was dissolved in 1–2 ml 0.10 M NaOH and sonically dispersed for 1 h at room temperature. One aliquot was removed for protein determination, and a second for the determination of total radioactivity by scintillation counting. Unlabeled HPB was added as an internal standard, then the solution was neutralized and the precipitated globin was removed by centrifugation. The supernatant was extracted 3 times with methylene chloride, which was then evaporated to dryness. The residue was dissolved in H_2O and analyzed by HPLC (system I).

Analysis of Methylated Amino Acids. Globin (5–10 mg) was hydrolyzed in 1.0 ml constant boiling 6 N HCl at 120°C for 18–24 h. The hydrolysis was performed in 5-ml vacuum hydrolysis tubes (Pierce). The 6 N HCl was removed, and the amino acids were derivatized with phenylisothiocyanate (Ref. 19, The Picotag Method, Waters, Milford, MA). The amino acids were separated by HPLC analysis on an Alltech Econosphere C_8 column (4.6 x 250 mm, Deerfield, IL). Solvent A was 0.5 M ammonium acetate, pH 6.8, and solvent B was 0.5 M ammonium acetate, pH 6.8, in 80% methanol. The amino acids were eluted as follows: from 0–12 min solvent A was decreased linearly from 100 to 80% and solvent B increased to 20%, from 12–30 min the percentage of solvent A to solvent B decreased linearly to 60% A:40% B, then from 30–60 min to 40% A:60% B. The flow rate was 1 ml/min and the elutant was monitored for UV absorbance at 254 nm and for radioactivity.

Methyl esters of aspartic and glutamic acid are unstable to 6 N HCl hydrolysis. The stability of other methylated amino acids to these conditions was determined. Greater than 90% of the *O*-methyltyrosine and *O*-methylserine were converted to tyrosine and serine, respectively. *S*-Methylcysteine, *N*-ε-methyllysine, and 1- and 3-methylhistidine were stable.

Separation of α and β Chains of Hemoglobin. Globin prepared from hemoglobin treated with either [5-^{3}H]NNK or [C^3H_3]NNK was separated by HPLC into its α and β chains (20). A solution of hemoglobin (2 mg/ml) was

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injected on a Vydac 214TP4 C_4 column (4.6 x 250 mm, Krackler Scientific, Inc.). Solvent A was 80% H_2O:20% CH_3CN, 0.1% trifluoroacetic acid and solvent B was 40% H_2O:60% CH_3CN:0.1% trifluoroacetic acid. The subunits of globin were eluted with a linear gradient form 45% solvent B to 65% solvent B in 70 min. The flow rate was 1 ml/min and detection was by UV absorbance at 254 nm. Two-min fractions were collected from 0 to 50 min, and the radioactivity present in each was determined by liquid scintillation counting.

RESULTS

The media from primary cultures of rat hepatocytes incubated with [5-^{3}H]NNK in the presence and absence of human RBC was analyzed by radioflow HPLC. As a control, RBC alone were incubated with [5-^{3}H]NNK and the media were analyzed. Representative chromatograms from 2-h incubations are presented in Fig. 2. The arrows indicate the elution times of synthetic metabolite standards. The data for the hepatocyte incubations are quantified in Table 1, for 2- and 18-h incubations. Both hepatocytes and RBC alone reduced NNK to NNAL. The major product of NNK α hydroxylation produced by rat hepatocytes was OPBA. HPB was also formed but to a much smaller extent. The radioactive peak coeluting with HPB was determined to contain no OPB. This aldehyde coelutes with HPB in HPLC systems I and II. A relatively large amount of 4-hydroxy-4-(3-pyridyl)-1-butyric acid was produced. This is the product of α hydroxylation of NNAL.

Surprisingly, when [5-^{3}H]NNK was incubated with RBC alone, OPBA (150 pmol/ml RBC, at 2 h) was released into the medium. The identity of this peak was confirmed by analysis with the use of two different HPLC systems. No other products of α hydroxylation were detected.

In 2-h incubations with hepatocytes and RBC, the amount of NNAL formed increased compared to that formed by hepatocytes alone (Fig. 2). This increase is probably due to the presence of carbonyl reductase in RBC. In addition, the amount of HPB decreased while a new peak appeared at 32 min and the peak at 34 min increased. The former coeluted with 4-hydroxy-1-(3-pyridyl)butanol and the latter did not coelute with any of the standard metabolites included in these analyses. After 18 h the relative amount of this unknown peak increased 13-fold. The identity of this peak was further investigated.

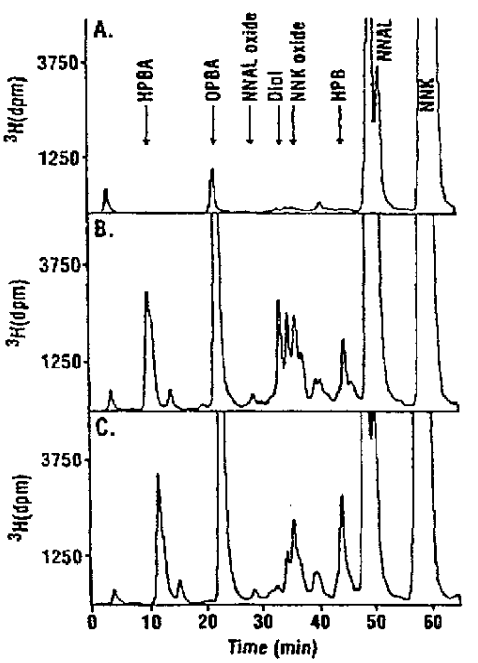

Fig. 2. Radioflow HPLC chromatogram of 5 μM [5-^{3}H]NNK metabolites released into the media after a 2-h incubation with, *A*, human RBC only; *B*, human RBC + rat hepatocytes; *C*, rat hepatocytes only. The *arrows* indicate the elution of metabolite standards (HPLC system I, 100 μl injection). *HPBA*, 4-hydroxy-4-(3-pyridyl)-1-butyric acid; *Diol*, 4-hydroxy-1-(3-pyridyl)butanol.

Table 1 *Metabolites of NNK formed by rat hepatocytes in presence or absence of RBC*[a]

Metabolite[b]	Hepatocytes only (n = 2)[c] 2 h	Hepatocytes only (n = 2)[c] 18 h	Hepatocytes + RBC (n = 4) 2 h	Hepatocytes + RBC (n = 4) 18 h
HPBA[d]	122	776	130 ± 14	1030 ± 140
OPBA	480	2120	491 ± 37	1368 ± 56
NNAL-*N*-oxide	6.5	170	11 ± 1.8	191 ± 21
Diol (32 min)[e]	12.1	151	70 ± 5	672 ± 60
Unknown (34 min)[e]	29.8	419	57 ± 5	746 ± 38
NNK-*N*-oxide[e]	83.2	412	108 ± 13	446 ± 47
HPB	86.2	119	36 ± 5	26 ± 4
NNAL	568	296	777 ± 41	383 ± 115
NNK	3680	300	3470 ± 67	266 ± 128

[a] Rat hepatocytes (3 × 10^6 cells/dish) in Williams' media at 37 °C were incubated with 5 μM NNK (1.84 Ci/mmol) for 2 or 18 h. For each time point 2 dishes were incubated with hepatocytes only and 4 dishes with hepatocytes plus 1 ml washed human RBC(mean ± SD).

[b] The media were analyzed for metabolites (pmol/ml media) by radio flow HPLC, system I, as described in "Materials and Methods," and illustrated in Fig. 2.

[c] The values are the average of 2 determinations, which agreed within 10%, except NNAL oxide at 2 h, the values were 8.1 and 5.0 pmol/ml.

[d] HPBA, 4-hydroxy-4-(3-pyridyl)-1-butyric acid; Diol, 4-hydroxy-1-(3-pyridyl)butanol.

[e] The radioactive peaks coeluting with these standards overlapped, therefore the values listed are estimates.

Previously Morse et al. (21) reported the presence of a glucuronide of NNAL in the urine of rats administered NNK. This metabolite eluted just prior to NNK-*N*-oxide as did our unknown. The HPLC system used in that study was the same as that used to obtain the chromatogram in Fig. 2. We were able to clearly separate the radioactive peaks coeluting with 4-hydroxy-1-(3-pyridyl)butanol and NNK-*N*-oxide from the unidentified peak by using a different C_{18} HPLC column with a modified gradient (Fig. 3, *A* and *B*). When the media from these incubations were treated with β-glucuronidase prior to HPLC analysis, the peak at 32.5 min (our unknown) disappeared and the NNAL peak increased a corresponding amount (Fig. 3*C*; Table 2). Therefore, the unknown has been identified as a glucuronide of NNAL. More of this conjugate was present during coincubations of hepatocytes with RBC (Table 1, unknown peak). This is most likely due to the additional NNAL formed by RBC. A small unknown peak, eluting at 27 min also disappears after β-glucuronidase treatment and the amount of HPB increases (Fig. 3). The HPB peak was collected and determined to contain no OPB.

Globin from RBC which were incubated with [5-^{3}H]NNK in the absence or presence of hepatocytes was isolated and analyzed for HPB released by base treatment. The only radioactive metabolite detected after base treatment of globin from RBC coincubated with hepatocytes was HPB. No [5-^{3}H]HPB was released from globin isolated from RBC treated with [5-^{3}H]NNK in the absence of hepatocytes. These data are presented in Fig. 4 and Table 3. To assure that the globin was free of unbound HPB formed by the hepatocytes, a portion of the dialyzed hemoglobin solution was concentrated on an Amicon membrane filter and the filtrate was analyzed for HPB by radio flow HPLC. No HPB was detected.

Interestingly, a significant amount of radioactivity was covalently bound to the globin obtained from RBC incubated with [5-^{3}H]NNK in the absence of hepatocytes. To further investigate the nature of this binding, RBC or freshly isolated hemoglobin solutions were incubated with either [C^3H_3]NNK or [5-^{3}H]NNK. Using RBC or a hemoglobin solution from rat or human, the amount of total tritium bound to

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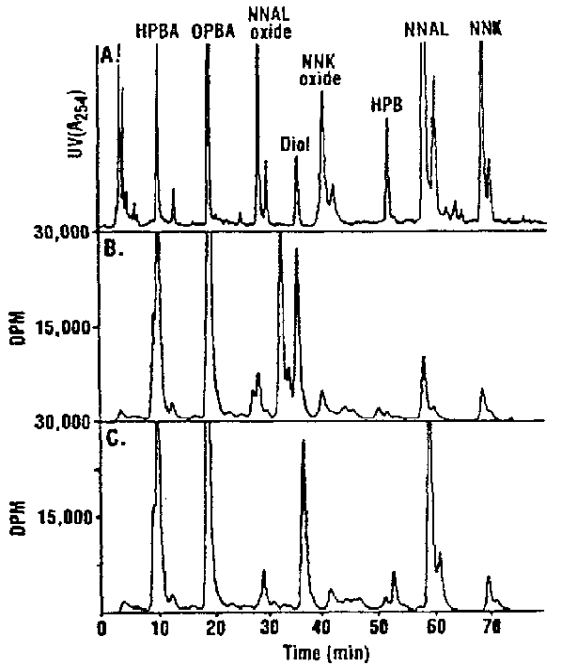

Fig. 3. Radioflow HPLC chromatogram of the medium from an 18-h incubation with hepatocytes, RBC, and 5 μM [5-^{3}H]NNK before (*B*), and after (*C*) β-glucuronidase treatment (HPLC system II; 100-μl injection). *A*, chromatogram with UV detection from a coinjection of NNK metabolite standards.

Table 2 *Effect of β-glucuronidase treatment on NNK metabolites formed during coincubation of rat hepatocytes and RBC with [5-^{3}H]NNK*[a]

Metabolites[b]	− glucuronidase	+ glucuronidase
HPBA[c]	920	1010
OPBA	1430	1330
NNAL-*N*-oxide	186	172
NNAL glucuronide	929	ND
NNK-*N*-oxide	172	133
Diol[c]	752	747
HPB	23	147
NNAL	311	1170
NNK	173	176

[a] One ml of media from each sample in Table 1 was incubated with 1 mg β-glucuronidase for 16 h at 37°C.

[b] The metabolites (pmol/ml media) were quantified by radio flow HPLC, system II described in "Materials and Methods" and illustrated in Fig. 3. The values in this table are for one sample, from an 18-h incubation of NNK with hepatocytes and RBC.

[c] HPBA, 4-hydroxy-4-(3-pyridyl)-1-butyric acid; Diol, 4-hydroxy-1-(3-pyridyl)butanol; ND, not detected.

globin was 3.7- to 10-fold greater with NNK tritiated in the methyl group than when the pyridine ring was tritiated (Table 4). The amount of tritium bound to human hemoglobin was less than that bound to rat hemoglobin for either [5-^{3}H]NNK or [C^3H_3]NNK. A number of factors could contribute to this; for example, different uptake of NNK by human and rat RBC and differences in the level of binding of NNK metabolites to rat and human hemoglobin. The latter would be the result of the different amino acid content of hemoglobin in these two species.

To confirm that the tritium was covalently bound, [^{3}H]globin was separated into its α and β chains and each was analyzed for the presence of tritium. The data presented in Fig. 5 are for hemoglobin treated with [C^3H_3]NNK. The radioactivity coeluted with the β chain as it did for [5-^{3}H]NNK-treated hemoglobin (data not shown). Interestingly, the radioactive peak from [5-^{3}H]NNK-treated hemoglobin eluted slightly earlier than that from [C^3H_3]NNK-treated hemoglobin. This is consistent with the radioactive modification of the globin being different for [5-^{3}H]NNK and [C^3H_3]NNK. That is, the tritium present is not due to bound NNK, but rather to attachment of different metabolites. One contains a [C^3H_3]methyl group and the other contains the [5-^{3}H]pyridine ring.

The presence of methylated amino acids in the [C^3H_3]NNK-treated hemoglobin was determined. Globin was hydrolyzed in 6 N HCl and the amino acids were analyzed as their phenylisothiocyanate derivatives by radio flow HPLC. Two major radioactive peaks were detected (Fig. 6). These peaks coeluted with 1-methylhistidine and *S*-methyl-

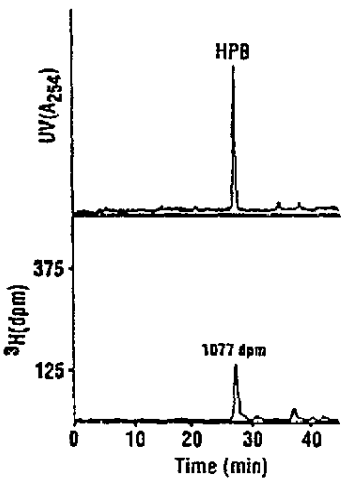

Fig. 4. Chromatogram obtained upon HPLC analysis of CH_2Cl_2 extracts of base-treated globin after coincubation of RBC with hepatocytes and 5 μM [5-^{3}H]NNK for 2 h (HPLC system III).

Table 3 *Levels of total binding and [5-^{3}H]HPB released from globin of RBC incubated with [5-^{3}H]NNK*[a]

Sample	HPB released[b]	Total binding[b]
Hepatocytes + RBC		
(n = 4) 2 h	6.35 ± 0.21	55.9 ± 2.7
(n = 4) 18 h	13.6 ± 3.6	218 ± 43
RBC only		
(n = 2) 2 h	ND[c]	56.7[d]
(n = 2) 18 h	ND[c]	229[e]

[a] RBC from 1 ml of human blood were incubated with 5 μM [5-^{3}H]NNK (1.84 Ci/mmol) with or without hepatocytes (3 × 10^6 cells) at 37°C in Williams' media. Globin was isolated (~100 mg/dish) and analyzed for total tritium bound or [^{3}H]HPB released by base treatment (details are described in "Materials and Methods").

[b] fmol/mg globin.

[c] ND, not detected (<0.5 fmol/mg).

[d] Average of 2: 58.7, 54.8.

[e] Average of 2: 232, 226.

Table 4 *Hemoglobin activation of NNK to a globin-binding species*[a]

Sample	Total binding (fmol/mg globin)[b]	
	[5-^{3}H]NNK[c]	[C^3H_3]NNK[d]
Rat RBC	440	4,880
Rat hemoglobin	1,200	11,500
Human RBC	150	548
Human hemoglobin	730	4,370

[a] Freshly isolated RBC or hemoglobin from 1 ml of blood were incubated with 5 μM NNK in Williams' media in an atmosphere of 95% O_2:5% CO_2 at 37°C for 18 h. Globin was prepared from each and analyzed for total tritium binding.

[b] Values are the average of the data obtained in 2 experiments. In each experiment globin was dissolved in 0.01 N HCl and the radioactivity present in 3 aliquots was determined by liquid scintillation counting.

[c] Specific activity, 2.1 or 1.34 Ci/mmol.

[d] Specific activity, 1.06 Ci/mmol.

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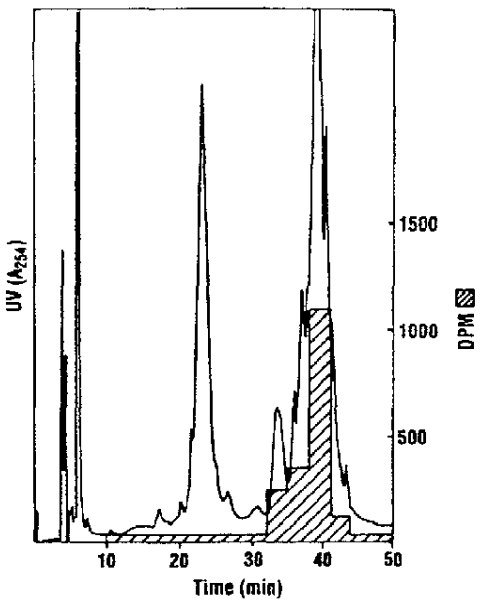

Fig. 5. HPLC chromatogram obtained on a C_4 reverse-phase column of [C^3H_3]NNK-treated rat hemoglobin. The α chains elute between 21 and 26 min and the β chain from 32 to 42 min.

cysteine and accounted for 30–50% of the total radioactivity in the sample. Ten to 15% of the radioactivity was lost upon 6 N HCl hydrolysis and concentration of the sample. The figure shown is for rat globin. The same result was obtained from human globin; that is, the only significant radioactive amino acids detected were 1-methylhistidine and *S*-methylcysteine in approximately the same amounts. No 3-methylhistidine (retention time, 29 min) was formed in rat or human hemoglobin.

DISCUSSION

In this study we have demonstrated the formation of the HPB-releasing hemoglobin adduct in RBC coincubated with hepatocytes and NNK. This adduct was not formed in the absence of hepatocytes. Base treatment of NNK-modified hemoglobin releases HPB from esters of aspartic or glutamic acid. The esters form from the reaction of 4-oxo-4-(3-pyridyl)butanediazohydroxide with hemoglobin (4, 12). Therefore, the hydroxynitrosamine *1* or the diazohydroxide *3*, formed in the hepatocyte, traveled out of this cell and into the RBC, where it reacted with hemoglobin.

Previously, Kim *et al.* (22) reported evidence that hydroxymethylmethylnitrosamine or methanediazohydroxide can penetrate the RBC membrane. In their study, 1- and 3-methylhistidine and *S*-methylcysteine were identified in hemoglobin of RBC that had been incubated with NDMA and rat liver microsomes. The ability of the methylating species to travel out of hepatocytes was demonstrated by Umbenhauer and Pegg (23). They incubated rat hepatocytes with NDMA in the presence of calf thymus DNA, and demonstrated the presence of O^6-methylguanine in the extracellular DNA. The data presented in our study are the first to provide evidence that an activated nitrosamine travels out of the cell in which it was metabolized and into RBC. This is analogous to what would happen *in vivo* to form a nitrosamine-derived hemoglobin adduct.

A second result of this study was the detection of NNAL glucuronide as a metabolite of NNK in hepatocyte cultures. At least one other glucuronide was released into the medium when hepatocytes were incubated with NNK (27 min; Fig. 3). Treatment with β-glucuronidase released HPB from this conjugate. This glucuronide was observed in incubations of hepatocytes alone and was more pronounced at lower concentrations of NNK, where the relative amount of NNAL formed is less (data not shown). This suggests the formation of a glucuronide conjugate of HPB or a glucuronide of the α-hydroxynitrosamine (Fig. 1). After β-glucuronidase treatment of the latter the resulting α-hydroxynitrosamine would decompose to HPB. The identity of this glucuronide is under investigation.

A completely unexpected outcome of our study on NNK hemoglobin adducts *in vitro* was our observation that RBC alone are capable of activating NNK to a hemoglobin-binding species. We propose that hemoglobin can metabolize NNK by α hydroxylation of the methylene carbon (Fig. 7). It has been reported by several investigators that hemoglobin has monooxygenase activity toward many different substrates. For example, hemoglobin mediates hydroxylation of aniline and other aromatic amines (24, 25), hydroxylation of cyclohexane (26), and epoxidation of styrene and 7,8-dihydroxy-7,8-dihydrobenzo-

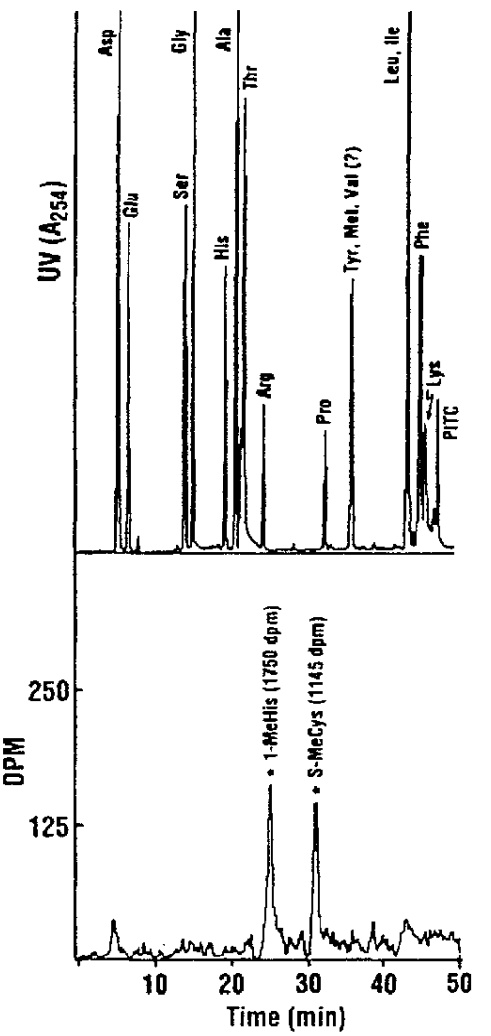

Fig. 6. Chromatogram obtained upon C_8 reverse-phase HPLC analysis, of 6 N HCl hydrolysate of rat [C^3H_3]globin. Prior to HPLC, amino acids were derivatized with phenylisothiocyanate. *Top*, UV detection of total amino acids in rat globin; *bottom*, radio flow detection of [C^3H_3]amino acids.

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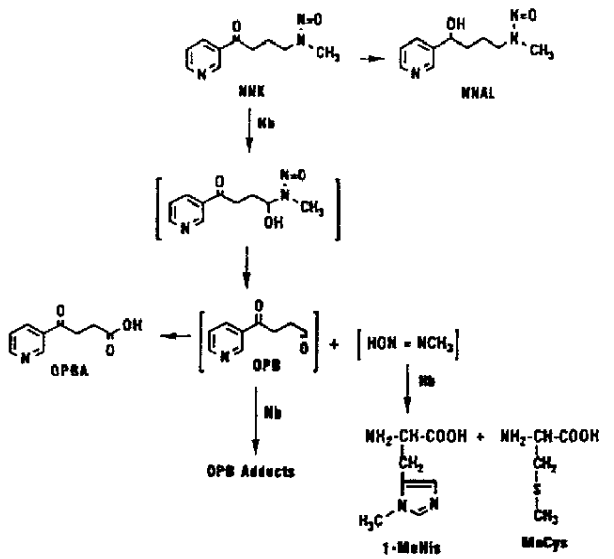

Fig. 7. Proposed pathway for RBC-mediated metabolism of NNK.

[*a*]pyrene (27–29). The mechanism by which these reactions occur is not well characterized. In most of these studies additional reducing agents such as P450 reductase and NADPH have been added. Catalano and Ortiz de Montellano (28, 29) have investigated the mechanism of epoxidation of styrene and 7,8-dihydroxy-7,8-dihydrobenzo-[*a*]pyrene by purified hemoglobin and H_2O_2. They concluded that H_2O_2 reacts with methemoglobin [Fe(III)] to generate a [Fe(IV)═O] protein radical. This radical can catalyze epoxidation by 3 different mechanisms: (*a*) ferryl oxygen transfer; (*b*) protein-mediated cooxidation; (*c*) proton abstraction, followed by the addition of H_2O.

In our studies on NNK activation by hemoglobin, no exogenous H_2O_2 was added. A small amount of H_2O_2 is generated in RBC. The autooxidation of hemoglobin produces methemoglobin and superoxide; the latter dismutates to yield H_2O_2 (30, 31). Therefore, the [Fe(IV)═O] protein radical could form from the methemoglobin and H_2O_2 generated by autoxidation of hemoglobin in the RBC. We propose that this radical would abstract a hydrogen from the methylene carbon of NNK as opposed to the methyl carbon. Recombination of the NNK radical with a hydroxy radical released from methemoglobin [Fe(III)—OH] would form the hydroxylated nitrosamine *1*. Methemoglobin would form from the [Fe(IV)═O] protein radical. This mechanism of NNK α hydroxylation is analogous to the pathway of NDMA metabolism proposed to involve an α-nitrosamino radical (32). As illustrated in Fig. 7, the hydroxylated nitrosamine decomposes to OPB and methanediazohydroxide. The former would further oxidize to OPBA, which was identified in the incubation media. No OPB was detected in the media. But there was a significant amount of tritium bound to hemoglobin of RBC treated with [5-^{3}H]NNK, which was not released upon base treatment. One source of these adducts may be the reaction of OPB with globin. Peterson[4] has characterized *N*-(5-amino-5-carboxylbutyl)-2-(3-pyridyl)-pyrrole as a stable product of the reaction of OPB with lysine. In future studies to characterize NNK hemoglobin adducts, both *in vivo* and *in vitro*, we plan to look for this modified amino acid.

We have identified *S*-methylcysteine and 1-methylhistidine as products of the reaction of NNK with hemoglobin. These methylated amino acids are formed from the reaction of methanediazohydroxide with these nucleophilic sites in hemoglobin. It is interesting that these were the major methylated amino acids detected. If the heme group is involved in the activation of NNK to methanediazohydroxide, β-cysteine 93 and histidine 92 are the closest nucleophiles.

Hemoglobin adducts are proposed as measures of the internal dose of the activated carcinogen. Therefore, the adduct should be a measure of an individual's capacity to metabolically activate the carcinogen. If hemoglobin is capable of activating a carcinogen to a particular adduct, then this adduct is not a good dosimeter. In the case of NNK, the HPB-releasing hemoglobin adduct is formed from a metabolite of NNK produced in hepatocytes which travels out of that cell and into RBC. But, other NNK hemoglobin adducts are formed by RBC in the absence of any exogenous activation system. Therefore, this second type of NNK adducts would not be good dosimeters, nor would the adducts of other carcinogens generated by a hemoglobin-mediated reaction.

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[4] L. A. Peterson, unpublished results.

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